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Streptomyces: A Screening Tool for Bacterial Cell Division Inhibitors

Charul Jani[1], **Elitza I. Tocheva**[3], **Scott McAuley**[2], **Arryn Craney**[1], **Grant J. Jensen**[3,4], and **Justin Nodwell**[1,2]

[1]Department of Biochemistry and Biomedical Sciences, Michael DeGroote Institute for Infectious Diseases Research, McMaster University, Hamilton, ON, Canada

[2]Department of Biochemistry, University of Toronto, Toronto, ON, Canada

[3]Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, USA

[4]Howard Hughes Medical Institute, Chevy Chase, MD, USA

Abstract

Cell division is essential for spore formation but not for viability in the filamentous streptomycetes bacteria. Failure to complete cell division instead blocks spore formation, a phenotype that can be visualized by the absence of gray (in *Streptomyces coelicolor*) and green (in *Streptomyces venezuelae*) spore-associated pigmentation. Despite the lack of essentiality, the streptomycetes divisome is similar to that of other prokaryotes. Therefore, the chemical inhibitors of sporulation in model streptomycetes may interfere with the cell division in rod-shaped bacteria as well. To test this, we investigated 196 compounds that inhibit sporulation in *S. coelicolor.* We show that 19 of these compounds cause filamentous growth in *Bacillus subtilis*, consistent with impaired cell division. One of the compounds is a DNA-damaging agent and inhibits cell division by activating the SOS response. The remaining 18 act independently of known stress responses and may therefore act on the divisome or on divisome positioning and stability. Three of the compounds (Fil-1, Fil-2, and Fil-3) confer distinct cell division defects on *B. subtilis*. They also block *B. subtilis* sporulation, which is mechanistically unrelated to the sporulation pathway of streptomycetes but is also dependent on the divisome. We discuss ways in which these differing phenotypes can be used in screens for cell division inhibitors.

Keywords

cell-based assays; in vivo screening; bacterial cell division; sporulation; *Streptomyces*

Corresponding Author: Justin Nodwell, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada. ; Email: justin.nodwell@utoronto.ca

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Introduction

Cell division is essential for viability in almost all bacteria. Its function is governed by the divisome, a multiprotein complex that ensures proper positioning and construction of the septum. Septum formation is followed by a cytokinesis event that separates the two daughter cells. This process responds to environmental signals and stresses to ensure that cell division does not occur under unfavorable conditions. For example, damage to genomic DNA or the cell wall can lead to the arrest of cell division, allowing repair mechanisms to be deployed.[1]

The divisome of the rod-shaped bacterium *Bacillus subtilis* consists of core proteins FtsZ, FtsA, FtsW, PBP1, PBP2B, EzrA, DivIB, FtsL, DivIC, and SepF, as well as other proteins serve to regulate divisome assembly and placement.[2] The divisome of the filamentous bacterium *Streptomyces coelicolor* is closely related, although it lacks FtsA.[3] Conserved enzymatic activities ascribed to the divisome so far include the GTPase activity of FtsZ, the ATPase activity of FtsA, the transpeptidase activity of FtsI, and the transport of lipid-linked cell wall precursors associated with FtsW.[4–6] The biochemical functions of the other divisomal proteins are less well understood, although it is clear that they contribute to divisomal stability and localization through a large number of protein-protein interactions.[2]

There is growing interest in the development of chemical probes of divisome function, most of which has been focused on FtsZ.[7–9] FtsZ is a tubulin-like protein that assembles at prospective division sites and forms a ring-like structure called the “Z-ring.” The Z-ring recruits the other cell division proteins, leading to the formation of a functional divisome. FtsZ forms polymers in vitro and also hydrolyzes guanosine triphosphate (GTP). These quantifiable activities have been used to develop in vitro assays for screening compound libraries, resulting in the discovery of the compounds such as PC190723, PC58538, viridotoxin, the zantrins, and others that interfere with cell division.[8–10] More recent work aimed at inhibiting the MipZ protein in *Caulobacter crescentus* led to the discovery of the compound divin, which blocks divisome assembly by a currently unknown mechanism.[11] Chemical inhibitors of the other divisome constituents would be valuable as probes of their function, and, given the essentiality of the divisome in most prokaryotes, these inhibitors could serve as lead compounds for the development of new antibiotics. However, because of a lack of quantifiable in vitro activities, and because the divisome is essential in most bacteria, it is difficult to screen for inhibitors of most of these proteins.

The *Streptomyces* life cycle is different from that of most other bacteria. Growth begins with spore germination and the formation of a vegetative colony of filamentous substrate hyphae. Following this, a layer of reproductive aerial hyphae grows up from the colony surface to form a white, fuzzy-looking aerial mycelium. Cell division is relatively rare in the substrate hyphae, resulting in cells composed of long chambers containing multiple chromosomes. In contrast, a developmentally regulated round of cell division takes place in the aerial hyphae that divides each filament into chains of uninucleoid compartments that subsequently develop into spores. In many streptomycetes, the last visually observable step in the life cycle is the deposition of a pigment in the maturing spores; this pigment is gray in *S. coelicolor* and green in *Streptomyces venezuelae*. This pigmentation serves as a phenotypic manifestation of the otherwise microscopic event of sporulation.[12] Mutations in the

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developmental genes that block the maturation of spores prevent the appearance of the pigments and are therefore referred to as *whi* for "white" because of the resulting white appearance of the aerial mycelium.[12] These visual cues make it possible to distinguish mutations that block the maturation of spores in those cells, including mutations that inhibit cell division.

In marked contrast to most other prokaryotes, cell division in *S. coelicolor* is not required for cell viability.[13,14] Previous work has shown that the *ftsZ* gene can be inactivated, eliminating the production of septa in both vegetative cells and aerial hyphae. While the resulting colony growth was clearly compromised, the cells were viable and could be grown in the laboratory.[13] In subsequent work, it was demonstrated that inactivation of a promoter element that upregulates *ftsZ* transcription in aerial hyphae blocks sporulation septation and confers a classic white phenotype. These colonies were healthier than those with fully inactivated *ftsZ*, presumably due to their ability to lay down occasional vegetative cross-walls in the substrate hyphae, demonstrating that the failure of sporulation septation prevents the expression of the spore pigment genes.[14] Cell division is therefore essential for the production of spores but not for viability, suggesting that the streptomycetes can provide unique insight into divisome function. In particular, we wondered whether chemical induction of a white phenotype could provide a method to identify compounds that inhibit cell division.

Here we describe proof of concept of a *Streptomyces*-based visual screening method for the identification of potential cell division inhibitors. In previous, work we visually screened 30,569 compounds against *S. coelicolor* and identified chemical inhibitors of several features of the organism's life cycle.[15] This included the discovery of 196 compounds that conferred a white colony phenotype reminiscent of the *whi* mutants. Here we have used these compounds to test the idea that chemical inhibition of *Streptomyces* sporulation can be a means of identifying compounds that compromise the action of the cell division apparatus in various bacteria, either directly or indirectly. We show that 19 of the 196 compounds cause filamentation in *B. subtilis*, the phenotype classically associated with a block in cell division in rod-shaped bacteria (**Suppl. Table S1**).[16] One of these compounds was determined to be a DNA-damaging agent and likely exerts its phenotypic effects on both *S. coelicolor* and *B. subtilis* through the SOS response. The remaining 18 compounds did not activate a DNA damage or cell wall damage stress response and therefore likely perturb the divisome or mechanisms that are important for its localization or stability. We explored three of these compounds—Fil-1, Fil-2, and Fil-3—in greater detail, showing that they confer distinct cell division phenotypes on *B. subtilis* and block endospore formation. We found that cells treated with these compounds had defects in septum placement and morphology at subinhibitory concentrations and that the compounds exhibit antibacterial activity.

Materials and Methods

Bacterial Growth and Culture

B. subtilis 168 was grown at 37 °C in Luria-Bertani (LB) media. *S. coelicolor* strains were grown in R5M, R2YE, or MS media, while *S. venezuelae* was grown in MYM media at 30 °C. A thiostrepton concentration of 30 μg/mL was used to induce *sulA* and *chiZ* gene

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expression. Mitomycin C was used at 2 μg/mL, while kanamycin and tetracycline were used at 50 μg/mL. *Escherichia coli* XL-blue strain was used for cloning purpose, and cells were grown on LB agar at 37 °C.

Cloning

Oligonucleotides used to amplify *sulA* from *E. coli* XL-blue and *chiZ* from *Mycobacterium tuberculosis* are listed in **Supplemental Table S2**. The amplified genes were digested with restriction endonucleases NdeI and KpnI and ligated into the vector pIJ6902 digested with the same restriction enzymes. The resulting constructs were transformed in *E. coli* ET12567 strain containing plasmid pUZ8002 and introduced into *S. coelicolor* by conjugation.

B. subtilis Filamentation Assay

To test the induction of filamentation, an overnight culture of *B. subtilis* was diluted to OD_{600} = 0.05, and test molecules were added to a final concentration of 10 μM. After 6 of incubation at 37 °C, the cells were observed under the light microscope as described below.

Induction of *lacZ*

Strain containing *dinC-lacZ* fusion YB5018 (*dinC18:: Tn917lac metB5 trpC2 xin-1* SPβ^{-} *amyE*$^{+}$) was kindly provided by R. E. Yasbin, and *liaI-lacZ* (Em trpC2 *liaI*::pMUTIN attSPβ) fusion strain was provided by *Bacillus* Genetic Stock Centre (BGSCID—1A980). The strains containing *lacZ* fusion were spread on LB agar containing 8 μg/mL X-gal, and molecules were spotted on the plate at various concentrations.

Microscopy

For fluorescence microscopy, cultures grown in the presence of the molecules were pelleted by centrifugation at 5000 rpm for 15 s and resuspended in 200 μL of saline (0.85% NaCl) with 0.5 μg/mL FM4-64 (Molecular Probes/Life Technologies, Eugene, OR, USA), 0.2 μg/mL DAPI (4',6-diamidino-2-phe-nylindole), or 2 μg/mL Van-FL (vancomycin-BODIPY conjugate from Molecular Probes/Life Technologies) and were incubated for 5 to 10 min in the dark. Cells were washed with saline once and mounted on microscope slides covered with a thin film of 1.5% agarose in water prepared using the gene frame (Thermo Fisher, Waltham, MA, USA). Fluorescent as well as differential interference contrast (DIC) images were acquired with a Hamamatsu, SZK (Japan), and an Orca ER-AG camera attached to a Leica (Solms, Germany) DMI 6000 B microscope and analyzed with IPLab software (BD Biosciences, Franklin Lakes, NJ, USA).

Electron Cryotomography

B. subtilis Δ*ponA* cells were grown in liquid LB medium supplemented with 0, 50, 83, 100, and 150 μM Fil-2. Electron microscopy (EM) grids were prepared by plunge freezing cells in nitrogen-cooled liquid ethane. Data were collected on an FEI Polara (FEI Company, Hillsboro, OR) 300-kV FEG transmission electron microscope equipped with a Gatan energy filter and a lens-coupled 4k × 4k UltraCam (Gatan, Pleasanton, CA). Samples were imaged at a dosage of 200 $e^{-}/Å^{2}$ with a defocus of −10 μm and a tilt range from minus;60 to

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+60 degrees. Three-dimensional reconstructions and segmentations were produced with IMOD.[17]

Determination of Minimum Inhibitory Concentration Values

B. subtilis 168 was inoculated into LB liquid media and incubated overnight at 37 °C. The culture was then diluted to an OD_{600} of 0.005 and various volumes of the molecules added to the bacterial suspension. The mixtures were then incubated overnight at 37 °C and the minimum inhibitory concentration (MIC) determined as the lowest concentration that inhibited visible growth.

B. subtilis Sporulation Assay

Sporulation of *B. subtilis* 168 was induced by suspension in resuspension medium. In summary, *B. subtilis* 168 was inoculated into LB and incubated with aeration overnight at 37 °C. The overnight culture was diluted to an OD_{600} of 0.1 and incubated with aeration at 37 °C until reaching an OD_{600} of 0.5. The cells were then pelleted, resuspended in resuspension media, and incubated at 37 °C overnight. t = 0 was determined as the time at which the sample was placed in the incubator following resuspension. The concentration-dependent effects of Fil-1, Fil-2, and Fil-3 were determined by adding different concentrations of the molecules immediately following initiation of sporulation. After overnight incubation, samples were heated at 80 °C for at least 30 min, serially diluted, and plated on LB agar.

Results

Inhibiting Cell Division Blocks Sporulation in *Streptomyces*

We first explored the perturbation of cell division in *S. coelicolor* with known genetic and chemical inhibitors. The *E. coli* gene *sulA* and the *M. tuberculosis* gene *chiZ* encode inhibitors of cell division that are expressed in response to DNA damage as part of the SOS response.[18] Both of these inhibitors act via conserved divisome components: SulA binds FtsZ while ChiZ binds FtsI/Q.[19,20] Since these divisomal proteins are conserved in the streptomycetes, we predicted that expressing *sulA* and *chiZ* in *Streptomyces* would block septation and sporulation. We therefore created expression constructs placing each gene under the control of a thiostrepton-inducible promoter and introduced the resulting constructs into *S. coelicolor*. As shown in Figure 1a, the expression of *sulA* and *chiZ* conferred a white phenotype while the empty vector had no effect on the gray pigment production in *S. coelicolor*. This phenotype was confirmed by scanning electron microscopy (Fig. 1a). Consistent with previous work,[13] these images indicated that inhibition of FtsZ (by SulA) and FtsI/Q (by ChiZ) blocked sporulation in *S. coelicolor* without compromising viability.

We then tested the effects of previously reported chemical inhibitors of cell division on *S. coelicolor* development.[15,21,22] As with genetic inhibition, treatment of *S. coelicolor* with berberine, totarol, and PC190723 resulted in formation of white colonies indicative of a block in sporulation (Fig. 1b).

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DNA damage blocks cell division in most prokaryotes through various mechanisms that are activated as part of the SOS response.[18,20,23] We compared the effect of mitomycin C, a known DNA-damaging compound, and the translation inhibitors tetracycline and kanamycin on colony phenotype in *S. venezuelae* (Fig. 1c). All the tested compounds exhibited antimicrobial effects as observed by the zone of inhibition; however, a subinhibitory concentration of mitomycin C conferred a white phenotype, whereas tetracycline and kanamycin did not. Using light microscopy, we found that this phenotype was accompanied by incomplete sporulation (Fig. 1c). In contrast, sporulation was normal in the presence of subinhibitory concentrations of tetracycline and kanamycin.

These data indicated that genetic and chemical inhibition of cell division either by direct perturbation of the divisome or by DNA damage prevents the appearance and maturation of spores in streptomycetes. This agrees well with previous work.[13] We note that this is the first time that DNA damage has been demonstrated to block spore development in a streptomycete.

Identification of Small Molecules That Inhibit Cell Division

In a previous compound screen against the *S. coelicolor* life cycle, we identified 196 molecules that impaired sporulation.[15] Given the close link between cell division and sporulation in the streptomycetes, we reasoned that some of these molecules might act by blocking cell division. To test this hypothesis, we applied these molecules to the Gram-positive, rod-shaped bacterium, *B. subtilis*, and observed their impact on cells using light microscopy. Abnormal cell division in *B. subtilis* results in long filamentous cells in contrast to their normal rod-shaped morphology. Of the 196 compounds that impaired sporulation in *S. coelicolor*, 19 conferred a filamentous phenotype in *B. subtilis* (**Suppl. Table S1**), indicating an effect on normal cell division functions. We focused our subsequent analysis on three of these compounds, which we have named Fil-1, Fil-2, and Fil-3. These compounds exhibited antimicrobial activity against *B. subtilis* cells with MICs of 33 ± 7, 58 ± 4, and 100 ± 10 μM, respectively.

The effects of treatment with Fil-1, Fil-2, and Fil-3 on *B. subtilis* were compared with the effects of subinhibitory concentrations of known antibiotics and with two molecules thought to interfere with divisome function (Fig. 2a). Vancomycin and ampicillin (which target the cell wall) and kanamycin and tetracycline (which target the ribosome) had no effect on cell length at subinhibitory concentrations. In contrast, trimethoprim and novobiocin, which respectively target thymine biosynthesis and DNA gyrase, caused a doubling or tripling of cell length over the time course of the experiment, consistent with the activation of an SOS response via compromised DNA synthesis. Similarly, berberine and 3-MBA, both thought to compromise divisome function, caused a doubling or tripling of cell length.[21] The effect of Fil-1, Fil-2, and Fil-3 was most striking: cell length was increased 4- to 7-fold, clearly a significant impact on cell length. This could be consistent with an induction of the SOS response or with the direct or indirect inhibition of cell division.

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SOS-Independent Inhibition of Cell Division

To determine whether any of the 19 compounds that induced filamentation in *B. subtilis* were DNA-damaging agents, we applied them to *B. subtilis* strain YB5018 (*dinC18::Tn917lac metB5 trpC2 xin-1* SPβ^{-} *amyE*$^{+}$), in which a *lacZ* reporter is under the control of the DNA damage-inducible promoter *dinC* (*D*NA *d*amage *i*nducible).[24] The formation of a blue zone in the presence of X-gal is evidence for the induction of the SOS response. We grew this strain on LB agar containing 8 μg/mL X-gal and spotted 2 μL of 1-mM solutions of each of the 19 compounds that induced filamentous growth in *B. subtilis*. Only one of the 19 compounds, MAC-0179833, induced the SOS response. This is consistent with the fact that this compound is the well-known DNA-damaging agent bleomycin (Fig. 2b).

Previously, antibiotics interfering with cell wall synthesis have been shown to induce the SOS response, which can lead to cell filamentation. To test if any of the molecules interfere with cell wall synthesis, we also tested them against a *B. subtilis* strain containing a *liaI-lacZ* reporter fusion. The *liaI* gene is induced during a stress response to cell wall damage or interference in cell wall synthesis.[25] Again, while *liaI* was induced by vancomycin, the remaining compounds had no such effect (Fig. 2b).

These data indicate that all but one of these compounds induce filamentous growth through a mechanism independent from the SOS response.

Fil-1, Fil-2, and Fil-3 Disrupt Cell Division at Different Stages

Since these molecules do not induce DNA or cell wall damage, we wanted to investigate their specific impact on cell membrane structure, chromosome segregation, and peptidoglycan synthesis during cell division. We therefore observed the impact of Fil-1, Fil-2, and Fil-3 on *B. subtilis* cells stained with FM4-64 and DAPI using light microscopy.

As shown in Figure 3, Fil-1–treated cells showed characteristic cell elongation along with complete inhibition of septa formation. Staining with FM4-64 showed distinct cross-walls in the untreated cells, but the same were absent in the cells treated with Fil-1. The compound also blocked chromosome segregation: blue fluorescently stained chromosomes showed a continuous, diffused localization along the length of filamentous cells (Fig. 3a—D, E, and F). The observations are consistent with a block in the early stage of cell division.

In contrast, chromosome segregation appeared unaffected in Fil-2–treated cells, but the abnormalities in septum formation were clearly evident (Fig. 3a—G, H, and I). FM4-64 staining revealed the elongated cells with irregularly placed cross-walls and occasional twists (Fig. 3a—G, H, and I). This phenotype suggests a role of Fil-2 in influencing the divisome function, placement, or cell envelope synthesis. The defects in these processes can often confer a similar cell-twisting phenotype.

Fil-3–treated cells showed little or no defect in the early stages of cell division such as chromosomal segregation or septum formation (Fig. 3a—J, K, and L). Rather, the filamentous cells appeared as long chains of normally growing cells having regular septation but were clearly blocked in cytokinesis (Fig. 3a—J, K, and L). These observations suggest

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defects in cell separation, possibly by inhibition or impaired localization of a peptidoglycan hydrolase.

We used a fluorescently labeled vancomycin probe, Van-BODIPY, to investigate any impact on peptidoglycan biosynthesis. Vancomycin is a glycopeptide antibiotic that binds to the D-ala–D-ala terminal end of the pentapeptide chain in peptidoglycan.[26] We stained cells treated with Fil-1, Fil-2, and Fil-3 with Van-BODIPY and compared the localization of peptidoglycan synthesis by fluorescence microscopy. Consistent with our observations of membrane staining with FM4-64, Van-BODIPY staining revealed the prevention of cross-wall formation by Fil-1, the induction of irregular cell wall formation and septation in Fil-2–treated cells, and normal septum formation in otherwise filamentous cells by Fil-3 (Fig. 3b).

These data suggest that Fil-1, Fil-2, and Fil-3 impair cell division at three different stages. Fil-1 blocks an early event in cell division, Fil-2 causes aberrantly placed divisomal function, and Fil-3 prevents cell separation.

Electron Cryotomography of *B. subtilis* in the Presence of Fil-2

Our light microscopy experiments showed that while Fil-1–treated cells completely lacked cross-walls and Fil-3–treated cells formed normal cross-walls, Fil-2 caused the formation of septa and partial septa having abnormal morphology and positioning. To further investigate the effects of this compound on cell wall formation at higher resolution, we collected cryotomograms in the presence of different concentrations of Fil-2 using the *B. subtilis* Δ*ponA* strain. This strain is narrower than other *B. subtilis* strains, making it a more suitable subject for cryotomography.[27] At concentrations of 50 μM and below, the growth rate of the cells appeared unaffected. Consistent with the antimicrobial activity of this molecule, at 83 μM, growth was impaired, and at concentrations above 100 μM, cells failed to grow altogether. We therefore examined cell morphology in the presence of 83 μM Fil-2. When imaged with light microscopy, the cells appeared filamentous, and FM4-64 staining showed the presence of irregular division septa as observed with wild-type *B. subtilis*.

Imaging of *B. subtilis* with electron cryotomography revealed several structural insights. In all imaged cells, we observed instances where the peptidoglycan was thinner or irregular in thickness and dissociated from the cytoplasmic membrane in the presence of Fil-2 (Fig. 4a,b, $n = 14$ of 14). Some regular septa were observed in the presence of Fil-2 (Fig. 4c, $n = 2$ of 14) similar to previously imaged vegetative cells.[27] In several cases, we found that the peptidoglycan surrounding the division septa appeared loose, suggesting that synthesis or maintenance of the membrane could have been affected by Fil-2 (Fig. 4d, $n = 3$ of 14). We observed a number of clearly aberrant division events, including abnormalities that appeared as membrane invaginations surrounded by a thin layer of peptidoglycan (Fig. 4e,f, $n = 5$ of 14). These results could be consistent with Fil-2 targeting a mechanism required for normal anchoring of the divisome to the inner cell surface or maintaining membrane integrity.

Fil-1, Fil-2, and Fil-3 Inhibit Sporulation in *B. subtilis*

B. subtilis is an endospore-forming bacterium, so its sporulation program is distinct from that of the exospore-forming streptomycetes; however, it does involve a developmentally regulated cell division event. The hallmark of this program is the formation of an

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asymmetrically positioned septum, creating a sporangium with a smaller forespore within a larger mother cell.[28] The forespore goes on to develop into a highly resistant endospore supported by the mother cell, which eventually dies. Although the outcome of the two cell division events is completely different, the machinery involved in septum synthesis is identical. Therefore, any molecule inhibiting the vegetative cell division via affecting the overlapping divisomal protein must influence the endospore formation as well.

On the basis of the earlier observation where we found Fil-1, Fil-2, and Fil-3 to be influencing cell division in vegetative cells, we hypothesized they might also affect sporulation in *B. subtilis*. We tested the effect of Fil-1, Fil-2, and Fil-3 on their ability to block sporulation in *B. subtilis* by determining the number of heat-resistant viable spores from cultures treated with increasing concentrations of each molecule. Consistent with their ability to impair vegetative septations, we observed that Fil-1, Fil-2, and Fil-3 could significantly decrease the number of viable spores in a concentration-dependent manner (Fig. 5). The effect of Fil-1 was most potent.

These data suggest that Fil-1, Fil-2, and Fil-3 prevent the normal functioning of the divisome so as to cause aberrant septation in vegetative and sporulating cells—these are both lethal events.

Discussion

The phenotypic manifestation of cell division in the streptomycetes provides a unique means of investigating the interactions of small molecules with the divisome and the factors that position and stabilize it. While division is relatively unimportant to the vegetative cells composing the *Streptomyces* substrate hyphae, it is essential to the normal completion of sporulation. Since successful sporulation is easily observed by colony pigmentation and is not a requirement for viability, we sought to determine whether the observation of a white phenotype in *Streptomyces* could serve as an assay for identifying compounds that interfere with cell division.

We found that of 196 compounds previously shown to block spore-associated colony pigmentation in *S. coelicolor*, 19 also caused a filamentous phenotype in *B. subtilis*, consistent with a block in cell division. One of these compounds clearly did so through a DNA damage-mediated SOS response. The remaining 18 acted independently of at least DNA damage and cell wall damage stress responses. Closer examination revealed that Fil-1 blocked an early step in cell division, impairing both the formation of septa and the segregation of the chromosomes. Fil-2 permitted normal chromosome segregation but caused the formation of misshapen and aberrantly localized membrane- and cell wall–containing structures in place of normal septa. Fil-3 permitted seemingly normal septation events but blocked cytokinesis. These data strongly support the idea that chemical inhibition of spore maturation in streptomycetes can be used as an enriching screen for inhibitors of diverse steps in the bacterial cell division process. In biochemical experiments, we found that none of Fil-1, Fil-2, or Fil-3 blocked either the GTPase activity or polymer formation by purified FtsZ protein (data not shown), implying that they act via other divisomal constituents or that they compromise divisome placement or stabilization.

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The effect of Fil-1, Fil-2, and Fil-3 on endospore formation in *B. subtilis* is also significant. It has been demonstrated previously that sporulation is impaired in the presence of cell division inhibitors.[9] The fact that each Fil molecule impairs both endospore formation and vegetative cell division is also consistent with a molecular target that is either part of the divisome (although clearly not FtsZ) or in the apparatus that positions or stabilizes it.

This work suggests that the streptomycetes life cycle is a powerful tool for identifying chemical inhibitors of cell division. We believe that these bacteria could be employed to screen further molecules in conjunction with a screen for growth inhibition of *B. subtilis*. For example, a direct screen for compounds that block the expression of the *whiE* genes that generate the gray spore pigment might be an efficient screening regimen. Use of a luminescent reporter to monitor gene expression would be a good way to conduct such an assay in high throughput.[29] Conversely, compounds that have been found to block growth in *B. subtilis* could be tested for effects on the sporulation cycle in a streptomycete as a means of narrowing in on divisome-targeting compounds. This approach would be particularly powerful when supplemented with genetic tests for the induction of stress responses to DNA or cell wall damage. This simple in vivo visual screening method may allow identification of inhibitors of cell division protein whose in vitro biochemical function is unknown. The compounds identified in this manner could be used as probes to better understand the divisome or for the development of novel antibiotics against pathogenic bacteria, particularly those exhibiting resistance to existing antibacterial compounds.

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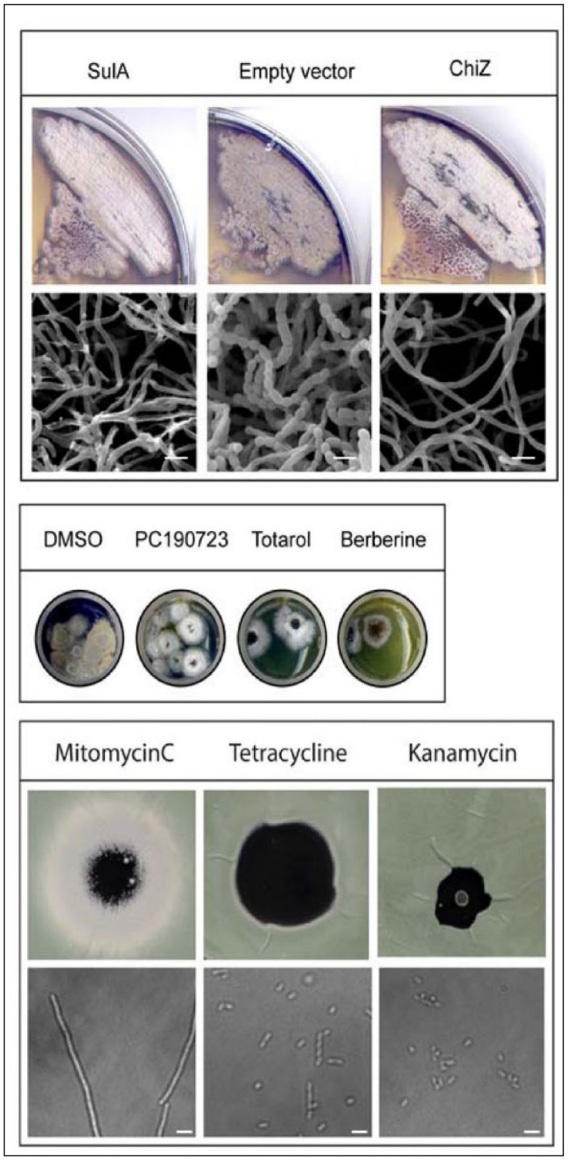

Figure 1.

The inhibition of cell division confers a white phenotype on *Streptomyces*. (**a**) The proteinaceous inhibitors SulA (FtsZ) and ChiZ (FtsI/Q) were expressed from the thiostrepton-inducible promoter. The colony morphology on solid media was observed after incubation at 30 °C for 4 days. The expression of *sulA* and *chiZ* resulted in inhibition of sporulation and formation of white fuzzy colonies. The empty vector containing strain underwent normal sporulation and produced gray-pigmented colonies. The lower panel shows SEM (scanning electron microscopy) images of the colony surface (scale bar = 2 μm). (**b**) Treatment of *S. coelicolor* with chemical inhibitor of FtsZ activity; PC190723, totarol, and berberine resulted in a sporulation block and the formation of a white colony, and the DMSO-treated colony appeared grey. (**c**) Mitomycin C, tetracycline, and kanamycin were spotted on *S. venezuelae* on solid media. In the upper panel, the black zone indicated absence of growth and the white zone indicated inhibition of sporulation. Indirect cell

division inhibition by the DNA-damaging antibiotic mitomycin C resulted in a sporulation block, whereas tetracycline and kanamycin had no effect on development. The lower panel shows the light microscopy images of the samples collected from the sublethal zone of the sample shown above (scale bar = 2 μm).

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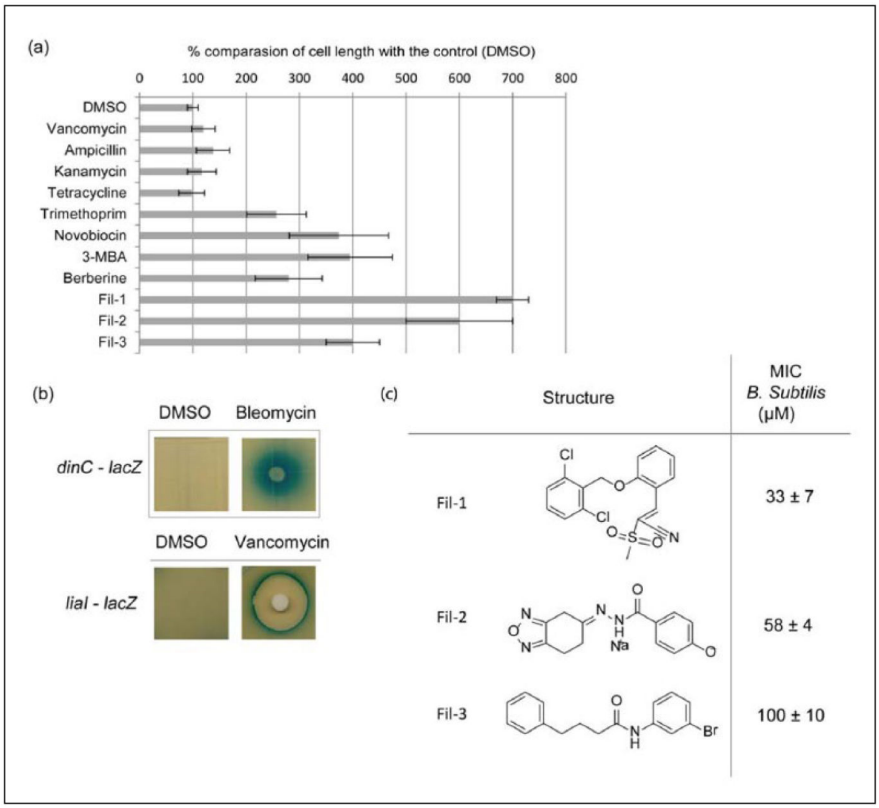

Figure 2.

Fil-1, Fil-2, and FIl-3 inhibit cell division in an SOS-independent manner. (**a**) *B. subtilis* cells were grown in the presence of cell wall–damaging antibiotics (vancomycin and ampicillin), translation-inhibiting antibiotics (kanamycin and tetracycline), inducers of the SOS response (trimethoprim and novobiocin), known FtsZ inhibitors (3-MBA and berberine), and Fil-1, Fil-2, and Fil-3 for 6 h at 37 °C. Changes in the cell shape were observed by light microscopy. Increased cell length in the presence of Fil-1, Fil-2, and Fil-3 suggests a block in cell division. (**b**) *B. subtilis* strain containing *dinC-lacZ* fusion was spread on solid media containing 8 μg/mL X-gal. Then, 2 μL of 10-mM molecules was spotted on the media. *lacZ* expression, seen as the blue zone, indicated induction of DNA damage. Fil-1, Fil-2, and Fil-3 did not induce the DNA damage. Bleomycin (MAC 0179833) induced the DNA damage, which can be seen as blue ring of *lacZ* induction. Similarly, strain containing *liaI-lacZ* fusion was used as an indicator of cell wall damage. Vancomycin is the positive control. Fil-1, Fil-2, and Fil-3 did not induce *liaI* expression, which indicates they may not damage the cell wall in *B. subtilis.* (**c**) Chemical structure of Fil-1, Fil-2, and Fil-3 and their minimum inhibitory concentration (MIC) against *B. subtilis* 168.

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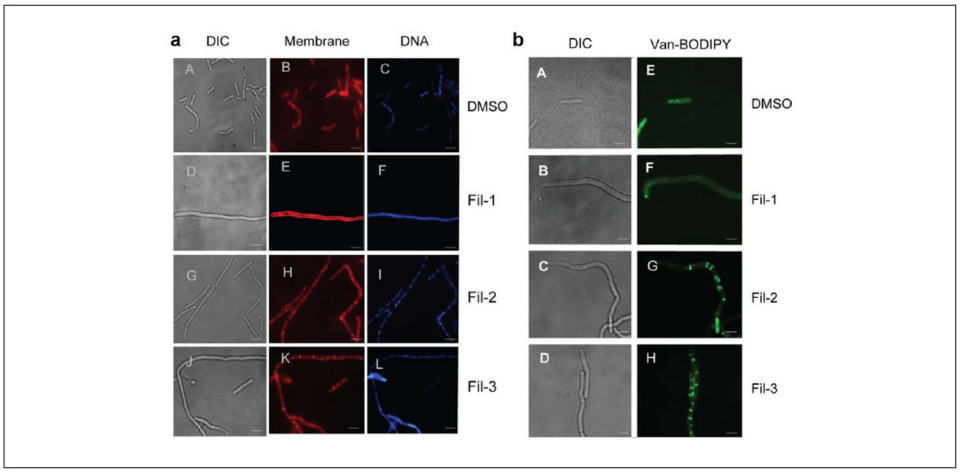

Figure 3.

Fil-1, Fil-2, and Fil-3 confer distinct cell division block on *B. subtilis*. (**a**) *B. subtilis* cells treated with Fil-1, Fil-2, and Fil-3 were stained with FM4-64 and DAPI to visualize the membrane and DNA. Fil-1–treated cells (D, E, and F) showed the absence of cross-walls and DNA segregation, Fil-2–treated cells (G, E, and H) showed the presence of irregular septa, and Fil-3–treated cells (J, K, and L) had no obvious defect in DNA segregation or septum formation but grew as chains. (**b**) To observe the effect of Fil-1, Fil-2, and Fil-3 on peptidoglycan biosynthesis in *B. subtilis*, cells treated with the molecules were stained with Van-BODIPY by incubating the cells with Van-BODIPY at room temperature for 5 min. DMSO-treated cells (A and E) accumulated fluorescent signal at the midcell, Fil-1–treated cells (B and F) showed absence of signal at the anticipated cross-wall formation sites, Fil-2–treated cells (C and G) showed the formation of irregular septa, and Fil-3–treated cells (D and H) showed the fluorescent signal at regular intervals, indicating normal synthesis of peptidoglycan (scale bar = 2 μm). DIC, differential interference contrast.

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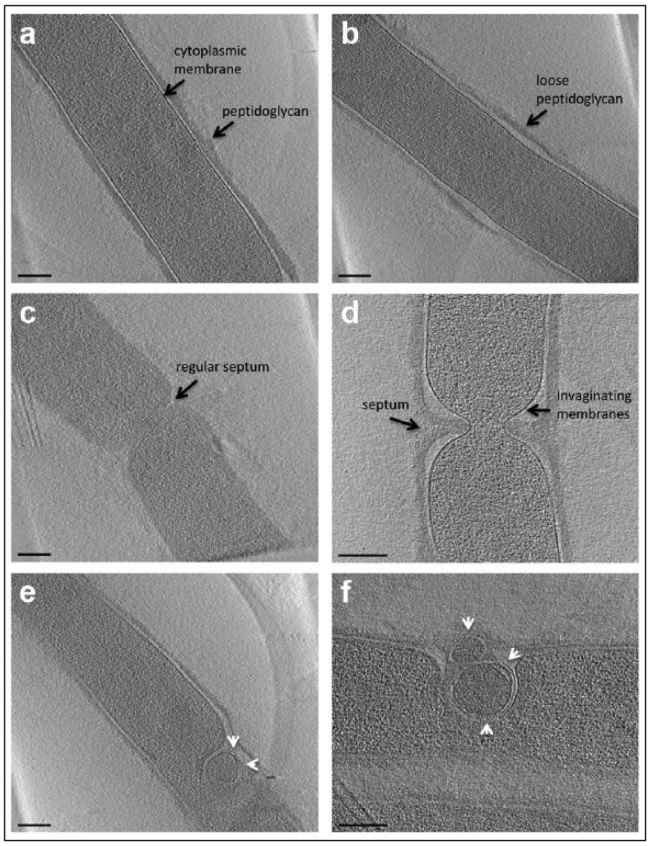

Figure 4.

Electron cryotomography of *B. subtilis* showing the effects of Fil-2 on cell morphology. (**a**, **b**) Tomographic slices through vegetative cells showing irregular and loose synthesis of peptidoglycan. (**c**) A regular-appearing vegetative septum. (**d**) Invaginating membranes appear separated from the peptidoglycan at the site of vegetative septa. (**e**, **f**) Irregular septa showing cytoplasmic membrane and peptidoglycan blebs suggestive of failed division sites (white arrowheads) (scale bar = 200 nm).

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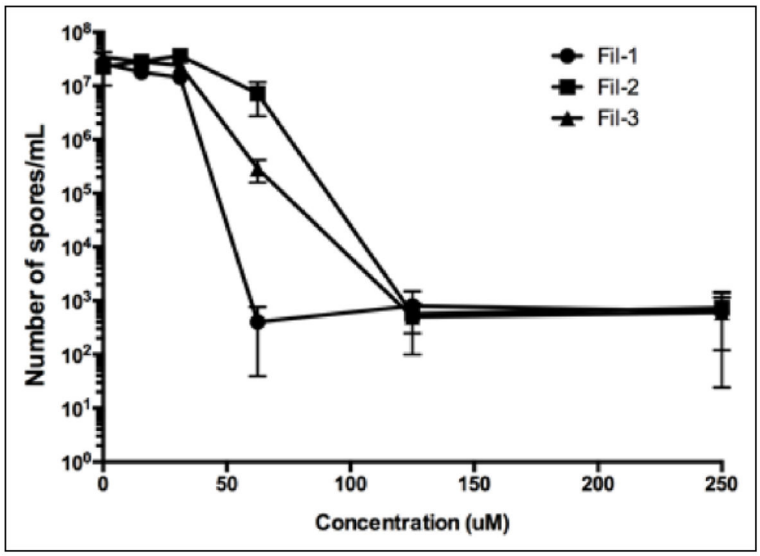

Figure 5.

Fil-1, Fil-2, and Fil-3 inhibit sporulation in *B. subtilis*. To determine whether Fil-1, Fil-2, and Fil-3 can influence endospore formation in *B. subtilis*, cells undergoing sporulation were treated with various concentrations of each molecule. Sporulation was initiated by resuspending the overnight grown culture of *B. subtilis* in the sporulation media. Immediately following resuspension, varying concentrations of the molecules were added and allowed to incubate overnight at 37 °C. Following 30 min of heat treatment at 80 °C to kill any remaining vegetative cells, the samples were serially diluted and plated to LB agar. After incubation, the colony-forming units were counted. A significant decrease in the number of heat-resistant spores was observed for all molecules in a concentration-dependent manner, with Fil-1 being the most potent. IC_{50} values for Fil-1, Fil-2, and Fil-3 are 41.4, 61.8, and 60.2, respectively.